Additional Mutations Detected in Sequential HIV-1 Isolates From ZDV-Treated Patients

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Sequential isolates from four patients under zidovudine (ZDV) therapy were obtained using PBMC coculture in the absence or in the presence of 0.25 μM ZDV. PBMC-based HIV susceptibility assay demonstrated the emergence of ZDV-resistance in the sequential isolates from the four patients. Except in one case, the isolates obtained in the presence of ZDV did not exhibit a greater resistance pattern than their counterparts obtained in the absence of the drug. In parallel, partial reverse transcriptase gene sequence was determined directly on amplified products from proviral DNA. In addition to the mutations previously described at the critical codons 41, 67, 70, 215, and 219, numerous additional mutations were found in either ZDV-sensitive or ZDV-resistant isolates. The mutation Thr215Tyr was not observed in a case of highly resistant virus (ZDV $IC_{50} > 6.25$ μM), while the mutation Lys70Arg was found in either resistant or sensitive ones. The analysis of additional mutations did not reveal any clear pattern for ZDV resistance but pointed out the existence of highly variable regions neighbouring the five critical codons. Neither nucleotide sequence nor PBMC-based susceptibility assay provided unambiguous data about pretherapy isolates or early on-therapy isolates which could predict the emergence of ZDV resistance in further samples. ***J Med Virol 51: 48–55, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: HIV-1 sequential isolates; ZDV resistance; RT coding region; additional mutations

INTRODUCTION

The development of various anti-HIV therapies results in the decrease of viral susceptibility to numerous drugs. Nucleoside drug mono-therapies–the most largely studied-have been associated with a only transitory effect and finally, a progression to disease. The theoretical likelihood of the emergence of resistance is increased with a large burden of replicating units, chronicity of infection, and underlying immunodeficiency. Each of these three conditions increases the number of replicative events in which virus mutations leading to resistance can occur. The existence of all these conditions in patients with advanced HIV infection prompted a search for mutants resistant to both nucleoside and non-nucleoside reverse transcriptase (RT) inhibitors: zidovudine (ZDV) [Larder and Kemp, 1989; Kellam et al., 1992] ddI [St. Clair et al., 1991], ddC [Fitzgibbon et al., 1992], nevirapine [Richmann et al., 1992; Nunberg et al., 1991], and others.

Treatment of individuals infected by HIV with ZDV was started in 1986 [Fischl et al., 1987; Fischl et al., 1990]. The first report on the emergence of ZDV-resistant strains was reported by Larder and Kemp [1989; Larder et al., 1989]. These reports were founded on the phenotypic and molecular analysis of HIV strains isolated from patients with AIDS or ARC who had received zidovudine therapy for at least 6 months. The decrease in ZDV sensitivity, expressed by means of ZDV IC_{50} and IC_{95} values, was associated with regularly occurring mutations at five codons of the reverse transcriptase (RT) gene which induced the following amino acids substitutions: Met41Leu, Asp67Asn, Lys70Arg, Thr215Tyr, or Thr215Phe and Lys219Gln. Despite the presence of these substitutions, RT was still functional [Larder and Kemp, 1989; Kellam et al., 1992]. Some authors [Boucher et al., 1992] determined an order for the appearance of these different mutations. During 2 years of ZDV treatment, the mutation at codon 70 of RT was observed first, but disappeared when the mutation at codon 215 of RT occurred. Consequently, the

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mutation at codon 70 reappeared in association with mutations at codons 67 and 41 and became stable, generating highly resistant viruses.

The experimental data have suggested that the stage of disease [Richmann et al., 1990], the duration of ZDV therapy [Larder et al., 1989; Richmann et al., 1990; Land et al., 1990], the number of mutations at specific codons of HIV RT gene (Larder and Kemp, 1989; Richmann et al., 1991; Boucher et al., 1992] were all associated with an increased likelihood of detecting phenotypically resistant strains in vitro. Since the level of resistance was reported to progress during therapy, the early detection of resistant viruses (especially in the samples collected before starting the treatment) might be beneficial for treated patients.

We have demonstrated previously that some isolates obtained in PBMC coculture from patients with long term ZDV therapy displayed ZDV resistance in a PBMC-based assay in vitro and that their genome had the Thr215Tyr mutation [Jung et al., 1992]. This was fully concordant with data obtained with MT2 isolates and gave strength to the assumption that the search for 215 mutation could be proposed as a general strategy at any stage of HIV infection.

We have therefore undertaken a study of sequential samples from four patients with long term ZDV therapy with two main questions (i) to see whether the presence of ZDV during virus isolation could enhance the detection of ZDV resistance and, even, predict its occurrence in further samples (ii) to check that the emergence and the increase of ZDV resistance was specifically associated with the appearance of a limited number of crucial mutations, with a prominent role of Thr215Tyr. The convergent goal of these two approaches was the definition of a novel strategy of anticipation and molecular detection of resistance for the monitoring of ZDV therapy.

MATERIALS AND METHODS

Patients

Four HIV-infected individuals were studied (Table I). All patients, previously untreated by any antiviral drug, started ZDV therapy at day 0 with a daily dose of 1 g. The monotherapy with ZDV was administered for an overall median period of 33 months and there was no addition of any other therapy. Three sequential PBMC samples were collected for patients E, K, and W: the first one just before starting ZDV therapy (baseline), the second after 16–25 months (period 1), and the third after 32–44 months of therapy (period 3). For patient R only the first two samples were available.

Isolation of HIV-1 From PBMC Samples

Three PBMC samples from each patient (only two from patient R), were cocultivated with phytohaemagglutinin-stimulated PBMC from healthy donors in order to obtain the virus isolates for ZDV susceptibility testing. Each PBMC sample was divided in two parts and cultivated either in the absence or the presence of 0.25 μM ZDV. Two viral isolates were then obtained from each PBMC sample and were designated as a combination of the donor initial, the duration of therapy at the time the sample was obtained, the presence of 0.25 μM ZDV during isolation culture. For example, E0 and E0z represented the two isolates from patient E, at initiation of ZDV therapy, obtained in the absence of ZDV and in the presence of 0.25 μM ZDV respectively. In the case of sample W32 the quantity of PBMC recovered after thawing was not sufficient to carry on two cell cultures and therefore a W32z isolate was not available. RT microassay was carried out on cell culture supernatants as already described [Schwartz et al., 1988]. During the primary isolation, RT peaks in culture supernatants were obtained after a median time of 19 days (range 11–26 days).

Determination of ZDV Sensitivity of Sequential HIV Isolates

The test was performed in 96-well culture plates following the consensus method of Action Coordonnee N°11 of Agence Nationale de Recherche sur le SIDA [Brun-Vezinet et al., 1992]. Briefly, the PBMCs from healthy blood donors were infected by undiluted virus, and four ten-fold decreasing viral concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). Zidovudine was added after viral adsorption in four final concentrations: 0.05 μM, 0.25 μM, 1.25 μM, and 6.25 μM. Each dilution of viral supernatant and each ZDV concentration were tested in quadruplicate. For viral growth assessment, the RT activity was measured at days 5 and 10 after infection. Two types of controls were included: four viral growth controls and eight negative controls, both in the absence of ZDV.

For each plate the RT activity cut-off value was defined as the mean value of RT activity measured in the supernatants of eight wells containing uninfected PBMC plus three standard deviations. The virus titer was determined in the absence of zidovudine, calculated according to the method of Karber, and expressed as tissue culture infectious dose ($TCID_{50}$) per ml of viral inoculum. The 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) were defined as the drug concentrations inhibiting 50% and 90% of the RT activity of 100 $TCID_{50}$ inoculum at day 10 after infection. The isolates were considered ZDV-sensitive if they exhibited the following characteristics: $IC_{50} \leq 0.05$ μM and $IC_{90} \leq 0.5$ μM. The partially resistant isolates exhibited the following characteristics: $0.05 < IC_{50} < 1$ μM and/or $IC_{90} > 0.5$ μM. The isolates were considered ZDV-resistant when $IC_{50} > 1$ μM.

Proviral DNA Preparation, PCR, and Determination of Nucleotide Sequence

The proviral DNA was prepared by standard phenol extraction and ethanol precipitation [Sambrook et al., 1989]. PCR was performed according to standard procedure described elsewhere [Saiki et al., 1988]. One microgram of purified DNA was amplified using primers MJ3 and MJ4, as already described [Jung et al., 1992]. The nucleotide sequence of reverse transcriptase of HIV iso-

TABLE I. Characteristics of the Four Patients Studied at the Time of Virus Isolation

Patient	ZDV therapy (months)	Antigen p24 (pg per ml)[a]	CD4 cells count (per μl)	ZDV dose (mg per day)	Clinical events	CDC classification group
E	0	620	336	1000	—	IIIA
	16	<50	268	500	—	IIIA
	44	>1000	321	500	—	IIIA
K	0	<50	343	1000	oral candidiasis	IVC2
	20	<50	173	500	—	IVC2
	24	<50	216	500	—	IVC2
R	0	<50	276	1000	thrombocytopenia	IIIB
	25	<50	113	750	thrombocytopenia	IIIB
W	0	<50	168	1000	herpes simplex labialis	III
	20	<50	152	750	—	III
	32	<50	185	500	herpes zoster	IVC2

[a]The cut-off value is 50 pg/ml.

lates was determined directly from amplified DNA with two separate primers: MJ5 for the RT region including the codons 41 and 70 and MJ6 for the RT region including the codons 215 and 219 [June et al., 1992]. The sequencing reactions [Sanger et al., 1977] were carried out with the T7 Polymerase Sequencing Kit (Pharmacia, Uppsala, Sweden) with [α-^{35}S]dATP [Candotti et al., 1991].

RESULTS

ZDV Susceptibility Profiles of HIV-1 Isolates

Table II shows the IC_{50} and IC_{90} values for all 21 HIV isolates. Seven out of eight pretherapy isolates, even those cultivated in the presence of 0.25 μM ZDV (samples E0, E0z, K0, K0z, R0, W0, and W0z, where "z" means samples obtained in the presence of ZDV in culture (see Materials and Methods) were uniformly sensitive, with their IC_{50} values lower than 0.05 μM ZDV and IC_{90} values lower than 0.5 μM ZDV. In the case of the patient R the presence of ZDV in cell culture during virus isolation (sample R0z) allowed us to select a partially resistant (PR) pretherapy isolate which exhibited a significant increase of IC_{90} value (IC_{90} = 0.89 μM ZDV) by comparison with its sensitive counterpart (R0), obtained without zidovudine in culture (IC_{90} < 0.05 μM).

In the group of period 1 isolates (16–25 months), four out of ten isolates (E16, E16z, W20, and W20z) remained sensitive to ZDV. In contrast, four isolates from patient K (K20, K20z, K24, and K24z) and two isolates from patient R (R25 and R25z) expressed a decrease in ZDV sensitivity. This was depicted by a concomitant increase of the IC_{90} values which ranged from 1.82 μM up to ≥ 6.25 μM and IC_{50} values which ranged from 0.33 μM up to 0.97 μM. All six isolates were classified as partially resistant.

The period 2 samples were studied in the case of two patients: E after 44 months and W after 32 months of therapy. The isolates E44 and W32 were partially resistant to ZDV while E44z was highly resistant to ZDV.

Considering all the results, the sensitivity assay we used detected 2- to ≥ 125-fold differences in IC_{50} and IC_{90} between pretherapy and on-therapy isolates. A clear progression to resistance was seen during therapy as established by the concomitant increase of both IC_{50} and IC_{90}. The selective concentration of zidovudine (0.25 μM) did not inhibit virus isolation from any PBMC sample even in the case of ZDV-sensitive isolates (E0z, K0z, W0z) but delayed virus isolation as compared to isolation in the absence of drug (data not shown). In two cases (E44/E44z and R0/R0z) the presence of ZDV permitted the isolation of a virus subpopulation more resistant than the predominant virus population present in the sample and isolated in the absence of ZDV. In the case of E44/E44z isolate pair, the presence of highly resistant viruses was yet suggested by the high IC_{90} value of E44 compared to its IC_{50} value. Such difference between IC_{50} and IC_{90} was not observed for other on-therapy isolates, suggesting that the emergence of a highly ZDV-resistant clone was not the major driving

TABLE II. ZDV Susceptibility Profiles of HIV Isolates

Isolate	IC_{50} (μM ZDV)	IC_{90} (μM ZDV)	Phenotype[a]
E0	<0.05	<0.05	S
E0z	<0.05	0.17	S
E16	<0.05	0.12	S
E16z	<0.05	0.21	S
E44	0.07	>6.25	PR
E44z	>6.25	>6.25	R
K0	0.05	0.19	S
K0z	0.05	0.11	S
K20	0.69	>6.25	PR
K20z	0.97	>6.25	PR
K24	0.45	2.24	PR
K24z	0.33	>6.25	PR
R0	<0.05	<0.05	S
R0z	0.08	0.89	PR
R25	0.48	4.22	PR
R25z	0.35	1.82	PR
W0	<0.05	0.08	S
W0z	<0.05	0.07	S
W20	<0.05	0.08	S
W20z	<0.05	0.16	S
W32	0.12	1.74	PR

[a]S, Sensitive (IC_{50} ≤ 0.05 μM and IC_{90} ≤ 0.5 μM ZDV); PR, partially resistant (0.05 < IC_{50} ≤ 1 μM and/or IC_{90} > 0.5 μM ZDV); R, resistant (IC_{50} > 1 μM ZDV).

TABLE III. Presence of Mutations at the Five Critical Codons of RT Gene

Isolate	Phenotype	Mutations at critical codons[a]				
		Met41Leu	Asp67Asn	Lys70Arg	Thr215Tyr	Lys219Gln
E0	S	−	−	−	ND	ND
E0z	S	−	−	−	−	−
E16	S	−	−	−	−	−
E16z	S	−	−	+	−	−
E44	PR	+	−	−	−	−
E44z	R	+	+/−	+/−	−	−
K0	S	−	−	−	−	+/−
K0z	S	−	−	−	−	+/−
K20	PR	−	−	−	+	+/−
K20z	PR	−	−	−	+	+/−
K24	PR	+	+	+	−	+/−
K24z	PR	+	+	−	−	+/−
R0	S	−	−	−	−	+/−
R0z	PR	+	−	−	−	+/−
R25	PR	+	−	−	+	+/−
R25z	PR	−	+/−	−	+	+/−
W0	S	−	−	−	−	+/−
W0z	S	−	−	−	−	+/−
W20	S	−	−	+/−	−	+/−
W20z	S	−	−	+/−	−	+/−
W32	PR	−	−	+	−	+/−

[a]+: Presence of the mutation indicated; − absence of the mutation; +/− presence of the sequence mixture including the mutation indicated; ND, not determined.

force in the appearance of overall ZDV-resistance for the four subjects studied.

Genetical Analysis of HIV Isolates

Proviral RT sequences from primary isolates were analyzed by direct sequencing of PCR products as described in Materials and Methods.

Firstly, we looked for the presence of specific mutations described by Larder and Kemp [1989] and Kellam et al. [1992] and their association to resistance to ZDV in vitro (Table III). None of the four mutations at critical codons 41, 67, 70, and 215 was found in pretherapy sensitive isolates except in the case of the partially resistant isolate R0z for which the modification Met41-Leu was detected. As far as Lys219Gln mutation was concerned, its presence was detected in many pretherapy isolates as sequence mixtures; this could be questioned in some cases due to a persistent sequence ambiguity at this codon reflecting either a complex quasi-species mixture or a sequencing point failure. In on-therapy isolates, all four mutations Met41Leu, Asp67Asn, Lys70Arg, and Thr215Tyr were found in different combinations. In many cases, isolates corresponded to mixtures of viruses which differed from each other by the presence of these four critical mutations. Moreover, the mutation Lys70Arg was present either in sensitive or in resistant isolates. The mutation Thr215Tyr was only present in resistant isolates but the most resistant isolate (E44z) did not contain this mutation. Regarding the presence of these mutations over time and influence of zidovudine selection pressure during isolation, no clear picture emerged from the follow up study. In six cases, mutations were detected at the same time with or without selective ZDV pressure: Met41Leu in E44/E44z and K24/K24z; Asp67Asn in K24/K24z; Lys70Arg in W20/W20z; Thr215Tyr in K20/K20z and R25/R25z. In the case of patient R, the detection of Met41Leu in R0z anticipated the detection of this mutation in R25. Surprisingly, this mutation was not present in R25z. In the case of patient W, the presence of mutation Lys70Arg in the part of the viral population W20/W20z anticipated the full presence of this mutation in W32. On the other hand, the mutation Thr215Tyr present in K20/K20z was absent in the two further isolates K24/K24z.

Secondly, we looked for the presence of mutations at the codons 41, 67, 70, and 215 distinct from those previously described (Table IV). In these five critical codons we observed the following additional modifications: Met41Trp, Asp67Gly, Lys70Gly, Thr215Asn, Thr215Gly, Thr215Trp, Lys219Cys, Lys219Ser, and Lys219Tyr. The Asp67Gly and Lys219Cys were observed when comparing the pretherapy isolates to the reference strain HIV-1 Lai. In sensitive on-therapy isolates we observed the presence of Lys70Gly, Thr215Trp, and Lys219Ser or Lys219Tyr. Three modifications were observed in resistant on-therapy isolates as compared to sensitive counterparts: Asp67Gly, Thr215Asn, and Thr215Gly. Only one, Asp67Gly, was selected during the isolation in the presence of ZDV. These results indicated that the modifications of crucial codons 41, 67, 70, 215, and 219 occurring during ZDV therapy were not restricted to those reported previously. However, the causative role of these additional mutations cannot be established unambiguously in the occurrence of ZDV resistance.

Bearing in mind the idea of high genetic variability of crucial codons, we looked for other mutations in the regions surrounding these codons. Numerous muta-

TABLE IV. Cumulated Analysis of Amino Acids Changes in Codon Regions 41–70 and 209–225

Codon number	Amino acid (Lai)	Amino acids substitutions when comparing pretherapy isolates to: Reference HIV-1 Lai strain	Sensitive on-therapy counterparts	Resistant on-therapy counterparts	Amino acids substitutions when comparing isolates obtained in the presence of ZDV to those obtained in the absence of the drug
41	Met	—	—	Leu	Leu, Trp
48	Ser	—		Gly	Gly
51	Gly	—	Ser, Cys	—	—
53	Glu	—	—	Asp, Lys	Lys
55	Pro	—	—	Ser, Thr	Ser, Thr, Leu
56	Tyr	Cys	—	Ser	—
57	Asn	Lys	—	—	—
59	Pro	Ser	—	Ser, Gly, Arg	Ser, Gly, Arg, Leu, Lys
60	Val	Leu, Ile	—	—	—
61	Phe	—	—	Ser	Ser
62	Ala	—	Pro	Gly, Val	Pro, Gly
67	Asp	Gly	—	Asn, Gly	Asn, Gly
68	Ser	—	—	Thr	Thr
69	Thr	—	Pro	Asn, Ile	Pro, Asn
70	Lys	—	Arg, Gly	Arg	Arg, Gly
209	Leu	—	—	Gly	Gly
210	Leu	—	Ser	His, Thr, Lys	His, Thr, Lys
211	Arg	Lys	Ala, Lys	Trp, Ile, Lys, Val, Asp	Asn, Lys, Asp
212	Trp	—	—	Leu, Val	Leu
213	Gly	—	Asn, Lys	Asn, Thr	—
214	Leu	Phe	Phe	Phe	—
215	Thr	—	Trp	Asn, Trp, Tyr, Gly	Trp
216	Thr	—	Ala	Ala, Pro	Ala, Pro
217	Pro	Leu	Ile, Arg	Gly, Ile, Gln, Ser, Arg	Gln, Arg
218	Asp	Cys	Ser, His, Cys	His, Cys, Gly, Tyr	His, Gly, Tyr
219[a]	Lys	Cys	Ser, Tyr	Ser	—
220	Lys	Ser	Trp, Tyr	Trp	Gln, Ser
221	His	—	Leu	Leu, Asn	Asn
222	Gln	—	—	Ile, Lys, His	Lys, His
223	Lys	Ile	Phe	Phe, Glu	Glu
224	Glu	Lys	Thr, Tyr	Tyr, Lys, Arg	Thr, Arg
225	Pro	Leu	Gly, Trp	Leu, Trp, Asp	Trp

[a]Six strains only have been examined.

tions were observed in the genome regions coding for codons 41–70 and 209–225. Some of them induced amino acid changes depicted in Table IV. These changes were more frequent and involved more amino acids in region 209–225 than in region 41–70. Some of these modifications were observed when comparing the pretherapy isolates to the reference strain HIV-1 Lai and can be interpreted as additional evidence of the well known HIV-1 variability from strain to strain: Arg211Lys, Leu214Phe, Pro217Leu, Asp218Cys, Lys220-Ser, Lys223Ile, Glu224Lys, and Pro225Leu. During ZDV therapy, all codons in the region 209–225 were subject to modifications. In sensitive on-therapy isolates we observed some modifications which were not present in on-therapy corresponding resistant isolates: Leu210Ser, Arg211Ala, Gly213Lys, Asp218Ser, Lys220Tyr, His221Asn, Glu224Thr, and Pro225Gly. Conversely, in resistant on-therapy isolates we detected 26 types of substitutions which were not present in on-therapy sensitive isolates: Leu209Gly, Leu210His, Leu210Thr, or Leu210Lys, Arg211Trp, Arg211Ile, Arg211Asp, or Arg211Val, Trp212Leu or Trp212Val, Gly213Thr, Thr216Pro, Pro217Gly, Pro217Gln or Pro217Ser, Asp218Gly or Asp218Tyr, His221Asn, Gln222Ile, Gln222Lys or Gln222His, Lys223Glu, Glu224Lys or Glu224Arg, Pro225Leu, and Pro225Asp. Among them, 15 were selected during isolated in the presence of ZDV. Comparable data, albeit at a lower extent, were obtained when analyzing the codon region 41–70 (Table IV for detailed results).

The conclusions of genetic analysis are summarized as follows: (i) changes of amino acids in regions 41–70 and 209–225 occurred during ZDV therapy, (ii) not all these changes were associated with resistant phenotype, many of them being associated with sensitive phenotypes, (iii) some changes associated previously with ZDV resistance such as Met41Leu and Thr215Tyr were confirmed to be present in the case of phenotypic resistance only, (iv) crucial codon changes reported previously by others appeared to belong to a more general set of changes corresponding to mutation-prone regions, (v) the selective presence of ZDV tended to select variants which differed from their counterparts obtained in the absence of drug by mutations occurring in the two regions but these mutations were not associated specifically with phenotypic resistance.

DISCUSSION

Sequential isolates from four patients under long term ZDV mono-therapy permitted examination of the emergence of ZDV resistance using both phenotypic and genotypic analyses. The aim was to confirm our previous findings [Jung et al., 1992] and to see whether some kind of prediction of ZDV resistance could be founded on sequential analysis of susceptibility to ZDV in vitro, and/or RT sequences. We observed the emergence of *in vitro* ZDV-resistant isolates in the four patients and detected numerous nucleotide changes in two regions of HIV-1 reverse transcriptase as well in ZDV-sensitive as in ZDV-resistant isolates.

Some of these modifications were consistent with previous findings and others were not. The Met41Leu substitution associated with ZDV resistance, was detected prior to treatment (in R0z) indicating the fact that ZDV-resistant clones preexist to treatment in HIV-infected subjects. The mutation at codon 67 was found in only four on-therapy resistant isolates (E44z, K24, K24z, and R25z), but we had previously demonstrated its presence in one sensitive HIV-1 isolate [Jung et al., 1992]. Two sensitive isolates, W20 and W20z, harboured the mutation Lys70Arg, which apparently did not confer any modification of their susceptibility to ZDV. This modification is thought to occur early in ZDV therapy but it has been detected prior to ZDV treatment [Jung et al., 1992; Zhang et al., 1991]. Therefore this amino acid modification corresponds to a spontaneous selection of naturally occurring mutants. A double mutation of codon 215 inducing Thr215Tyr substitution was found only in resistant isolates (K20, K20z, R25, and R25z) but was not in the most resistant isolate E44z, for which only three out of the four mutations at critical codons (41, 67, and 70) were observed. This is in disagreement with the common knowledge that this modification of codon 215 is a hallmark for ZDV resistance [Lacey and Larder, 1994]. Evidence was found for a Trp residue at codon 215 for this isolate and initially speculated that this might be responsible for the loss of ZDV sensitivity. However, the same amino acid was found at the same position in the sensitive isolates E16 and E16z. We now suppose that the high number of modified codons of isolate E44z in flanking regions of 215 codon and the modification of codon 215 itself interact for the loss of sensitivity of this isolate. The hypothesis is strengthened by the evidence of similar modifications in two pairs of resistant isolates: K20/K20z and K24/K24z. The former pair of isolates exhibited the critical mutation Thr215Tyr while the latter one exhibited a Thr215Gly change. Therefore, complex interactions between the critical codons and other neighbouring sites might be responsible for decrease of ZDV sensitivity rather than a single amino acid change.

We detected regularly the following additional mutations in on-therapy HIV isolates: Arg211Lys, Leu214-Phe, Thr216Ala, Thr216Pro, and Lys220Trp. The changes at codons 211 and 214 had already been observed, without any apparent association with decreased ZDV sensitivity [Levantis and Oxford, 1992; Levantis et al., 1993; Stein et al., 1994; our unpublished results]. Other authors reported recently [Sheehy and Desselberger, 1993] that their most resistant isolate, which grew in the presence of 15 μM ZDV, did not contain any of the published mutations associated with zidovudine resistance. The isolation of a highly mutated virus from a patient who underwent a long-term ZDV therapy was reported by Stein et al. [1994]. The virus had no change at positions 67, 70, 215, and 219 but showed some new changes at positions 62, 93, 210, and 214 only. A Leu210Trp mutation, not observed in our isolates, was reported in resistant isolates by others [Gurusinghe et al., 1995]. The isolation of a highly mutated viruses from several patients not subjected to antiretroviral therapy was also reported by Najera et al. [1995]. These viruses exhibited some of the mutations associated with ZDV, ddC, and d4T resistance. The substitutions observed by other groups in ZDV-resistant virus did not appear to be randomly distributed on the RT, but, which is consistent with our observations, clustered into two areas: amino acids 60–70 and 180–220. Mutations in one region were not necessarily accompanied by changes in the other region.

The role of additional mutations in the genesis of resistant phenotype requires more studies, especially in the case of a highly variable virus such as HIV. As yet, site-directed mutagenesis has been the most powerful approach to confirm the role of mutations in the decrease of sensitivity to ZDV. However, recombinant provirus HIV-1 DNA used in the experiments has to reflect as closely as possible the genetic background in which the additional mutations are suspected to act. This is a key point. The predominant role of the five critical changes studied by Larder and Kemp was suspected from the analysis of resistant MT2-adapted HIV-1 isolates from AIDS patients and confirmed by means of provirus reconstructions using an infectious clone derived from HTLV-IIIB strain, another T-cell-line-adapted virus. We suspect that the conclusion on the predominant role of the five mutations, particularly the 215 mutation, in ZDV resistance is absolutely correct for similar viruses, i.e., high/fast T-cell-line-adapted isolates from AIDS patients but cannot be extrapolated to any primary isolate from a treated subject at any stage of HIV infection. The major limitation for validating the role of additional mutations will be the need for infectious clones matching the genetic background of primary isolates as closely as possible. All these results might cast some restrictions to the rapid detection of HIV resistance to ZDV by means of PCR [Boucher et al., 1990] or PCR-LDR [Frenkel et al., 1995] since these approaches are targeted against a limited number of mutations considered as crucial.

Given the limitations of molecular analysis, it seems necessary to carry on phenotypic analysis of virus susceptibility. For all assays, we chose PBMC-based cell culture, since the rate of virus isolation from PBMC samples is approximately 90% in each case [Coombs et al., 1989; Ho et al., 1989; Rouzioux et al., 1991; Mayers

et al., 1992]. It has been confirmed that the use of PBMCs from different donors has no major effect on replication of various isolates of HIV-1 [Spira and Ho, 1995]. This conclusion was partly amended by the report of relative resistance to HIV-1 infection of purified CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures [Paxton et al., 1996]. Nevertheless, this recent finding did not question the general assessment that PBMCs are the most susceptible targets for most primary isolates of HIV-1. In the original study of Larder and Kemp [1989] on resistance to zidovudine [Larder et al., 1989] viruses were cultivated in transformed cell-lines MT2 and MT4. However, the use of these cell lines for viral sensitivity assays is of limited value, because it permits a successful isolation and characterization of only 30% of viruses. The use of PBMCs would then rule out the possible selection of HIV-1 clones in primary isolates obtained from cell line cultures which might introduce a bias and even simplifications in the study of ZDV resistance.

The HIV resistant strains, which emerge either in culture under ZDV or in vivo in ZDV-treated patients are supposed to be the genetic variants initially present as minor components in the virus population. Such variants arise and may be selected during treatment, since they develop faster than parental strains in the presence of RT inhibitors. In an attempt to detect resistant viruses as early as possible before and during ZDV therapy, we have carried out the virus isolation in presence of 0.25 μM ZDV, in parallel to standard virus isolation without the drug. In the presence of 0.25 μM ZDV, three pretherapy isolates were sensitive to the drug (E0z, K0z, and W0z) while only one pretherapy isolate had a partially resistant phenotype (R0z). Thus the anticipation of ZDV resistance by means of ZDV in culture medium for pretherapy isolates seems to be rather unsuccessful. It cannot be excluded that our ZDV concentration chosen was too low to select regularly the partially resistant HIV isolates in every pretherapy sample. However, 0.25 μM concentration was above the IC_{90} values obtained for sensitive isolates. This ZDV concentration was expected to inhibit more strongly the major sensitive part of viral population than for the minor resistant part. More stringent conditions, for example a ZDV concentration higher than 0.5 μM, might be used with the risk that the partially resistant viruses would be inhibited too. As reported recently [Stein et al., 1994], the selective concentration of ZDV should not exceed 5 μM since under these conditions of culture, it was impossible to isolate the virus from patients who have been receiving ZDV treatment for less than two months or from patients who have not been treated with ZDV. Mayers et al. [1992] reported the successful isolation of four post-therapy isolates in the presence of 4 μM ZDV. All isolates were ZDV-resistant and harboured at least one out of five critical RT mutations.

The limited predictive value of susceptibility assays is in agreement with some apparent discrepancies in the sequential occurrence of mutations in ZDV resistant isolates from the same individual. As discussed above, the modification of HIV susceptibility to ZDV does not seem to depend exclusively on five critical codons changes of the RT gene. In PBMC-based cultures we and others [Levantis et al., 1993; Stein et al., 1994; Sheehy and Desselberger, 1993; Gurusinghe et al., 1995] observed the presence of numerous additional mutations in the RT gene, suggesting multiple routes to loss of ZDV sensitivity. Due to this high diversity and technical limitations, as yet, resistance to ZDV cannot be approached as a continuous predictable process.

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